BACTERIOLOGICAL REVIEWS, June, 1966

Vol. 30, No. 2
Printed in U.S.A.

Symposium on In Vitro Studies of the Immune Response

III. Biochemical Agents Affecting the Inductive Phase of the Secondary Antibody Response Initiated In Vitro[1]

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INTRODUCTION

An old and obvious problem in immunology continues to be eludication of the steps in the immune response. Historically, an understanding of these steps was first approached by subdividing the response into several phases. The most useful such subdivision originated with the observation in 1908 that precipitin production in rabbits was depressed if X irradiation immediately preceded antigen injection (6). The optimal time of X irradiation has since been extensively studied and has led to the concept of an initial short radiosensitive period occurring during primary antigen stimulation and a subsequent long radio-resistant period concurrent with antibody synthesis (8). In the early 1950's, it was discovered that injection of corticosteroids produced a temporally similar type of immune suppression (7). More recently, many different kinds of metabolic inhibitors have been examined for their effects on antibody production [reviewed in (13, 14)]. Some of these have been tested clinically in the hope of finding an inhibitor which prevents the initiation of new antibody responses (for example, to a transplanted organ) without interfering with established antibody synthesis or other protein production. This desired property has focused attention on the so-called inductive phase, which encompasses all steps between the introduction of the antigen and the production of detectable antibody (19). This initial phase is followed by the long productive phase, which involves the final synthesis of antibody on ribosomes from amino acids and its release into the extracellular environment. This latter phase of antibody synthesis appears similar to that of other nonglobulin protein production, as has been illustrated in studies with cell-free systems prepared from lymph nodes or spleens of hyperimmunized animals (9, 18, 23).

During the inductive phase, the cellular multiplication and differentiation triggered by an antigen is probably somewhat staggered from one responding cell to another, so that the sequence of these steps is not precisely synchronous in a large population of antibody-producing cells. The dissection of these steps in an intact animal is further complicated by innumerable secondary influences on the immune response: cellular, hormonal, nutritional, and perhaps others as yet unrecognized. Many of these secondary influences are eliminated, or at least standardized to some degree, in tissue culture systems. One of the most useful of such systems involves the secondary response.

Initiation of the secondary antibody response in vitro was first reported in 1957 by Michaelides, who studied cultures of rabbit lymph node fragments. The important features of this system, including histology, were discussed in detail in two adjoining articles (10, 11). The great merit of the system is that it allows examination under cultural conditions of all phases of the secondary response. Other investigators had studied antibody production in tissue cultures, but their animals had received the antigenic stimulus

[1] A contribution to a symposium held at the Annual Meeting of the American Society for Microbiology, Atlantic City, N.J., 27 April 1965, with Abram B. Stavitsky as convener and Consultant Editor.

several days or so before the cultures were prepared [reviewed in (17)]. Thus the early phase of these responses was lost to direct study while the tissue remained in situ. But from our studies of the secondary response initiated in vitro, it now appears that this early phase (the inductive phase) is more sensitive to a variety of chemical agents than is the productive phase. These various agents undoubtedly affect different steps in the initiation of the response and thus represent possible metabolic tools for their elucidation. The work reviewed below concerns agents we have studied which affect mainly the inductive phase.

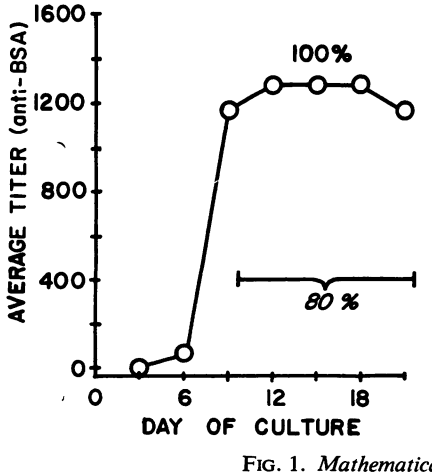
EXPERIMENTAL PROCEDURES

The general procedures employed in these experiments have been described by Michaelides and Coons (10) and Ambrose (2). Adult male rabbits received a primary stimulation in vivo to both bovine serum albumin (BSA) and diphtheria toxoid by means of a saline solution of these two antigens injected intracutaneously into the four toepads and two ears. Three to twelve months later, the lymph nodes draining the injection sites were excised and cut into 1-mm cubes. These pooled fragments received a secondary stimulation in vitro during a 2-hr incubation in culture medium containing 0.5 mg/ml of BSA and 5 Lf/ml of diphtheria toxoid. Excess antigen was washed from the fragments by several rinses with medium. Fourteen stimulated fragments were aligned in a Leighton tube and a thin pad of glass wool was inserted on top to hold them in place. Each tube received 1 ml of Eagle's Minimum Essential Medium (MEM) supplemented with five additional amino acids, vitamin B_{12}, penicillin, insulin, cortisol, and other substances described in each experiment. (Two experiments reviewed below antedate the development of the serum-free medium we currently use; the composition of the medium used in these experiments is listed in the relevant figures.) The cultures were kept stationary at 37 C for 15 to 21 days, during which time the culture fluids were replaced every 3 days. The fluids removed were immediately frozen and stored at −5 C. Antibody titers to BSA and diphtheria toxoid were subsequently measured by the hemagglutination method with tanned sheep erythrocytes.

Generally, from one previously primed rabbit, about 2 g of lymph node tissue can be obtained; from it, 80 to 100 culture tubes can be prepared, each containing about 25 mg of fragments. Three to four tubes are assigned to each variable in an experiment.

MATHEMATICAL TREATMENT OF TITRATION DATA

Figure 1 illustrates how we treat mathematically the titration data from the culture fluids. The graph shown on the left represents a typical secondary response initiated in vitro on day 0. The abscissa shows the time of the medium changes at 3-day intervals; the ordinate is a linear plot of the average hemagglutination titer. Six cultures were included in this group and were maintained for 21 days. The individual titers are listed to the right of the graph; the average titer for each medium change is given below. These data show that the response became measurable between day 3 and 6, reached a peak around day 12, and then remained at a high level of production for the next week. The shape of this



CULTURE TUBE #	DAY MEDIUM REMOVED 3	6	9	12	15	18	21
1	<10	40	640	1280	1280	1280	1280
2	<10	80	1280	1280	1280	1280	1280
3	<10	80	1280	1280	1280	1280	1280
4	<10	80	1280	1280	1280	1280	640
5	<10	80	1280	1280	1280	1280	1280
6	<10	40	1280	1280	1280	1280	1280
AVERAGE	0	67	1173	1280	1280	1280	1173

SUM:
DAY 0-21 ⟵ 6253 = 100% ⟶
DAY 9-21 ⟵ 5013 = *80%* ⟶

FIG. 1. *Mathematical treatment of the titration data; see text.*

response curve is influenced by the presence of serum in the medium, which invariably causes an earlier decline in antibody production than is seen with serum-free medium (3).

The graph in this figure also shows that most of the antibody appears after day 9. The quantitation of this fact illustrates also how we compare responses between different experimental groups. In this experiment, the sum of the average titers for the seven medium changes from day 3 through day 21 is 6,253 and is taken as the 100% reference response. The sum of the average titers of the media removed on day 12, 15, 18, and 21 is 5,013, which is 80% of the above reference value. Thus, 80% of the antibody produced during the 21 days of this experiment appeared in the culture fluids after day 9. (A higher percentage is often found in other experiments, as will be indicated in later figures.) In the case of other cultures in the same experiment which test a different variable, we determine the sum of their average titers over the course of the experiment. This number is compared with the control group's corresponding sum, which represents the 100% reference response; the per cent value is then calculated for the response permitted by this variable.

EVIDENCE FOR DE NOVO SYNTHESIS OF ANTIBODY

Inhibition by Puromycin and Incorporation of Labeled Amino Acids

Initially, we wondered when the antibody secreted over a 3-day period is actually synthesized. An answer is important here for interpreting much of our later data. Studies with puromycin and others involving the incorporation of labeled amino acids have led us to believe that the intracellular content of antibody in these cells is relatively small and that the transit time of the antibody (that is, the time from its synthesis until its secretion) is only several hours or less. The experiment shown in Fig. 2 concerns the effect of different concentrations of puromycin added to cultures for different intervals starting on day 9. In the reference, or control, response, 81% of the antibody produced appeared in the medium after day 9. The inclusion of 10 μM and 3 μM concentrations of puromycin in the media from day 9 through day 21 produced prompt, almost complete, suppression of synthesis. This suggested that antibody appearing in the culture fluids after day 9 is largely synthesized after that time.

The same conclusion was reached in more direct studies done recently with Frank F. Richards (*in preparation*). In these experiments, we employed ribonuclease-stimulated cultures prepared from rabbits previously injected with the antigen in Freund's adjuvant. Several months after the single ribonuclease injection, the lymph nodes were removed from the rabbit and were used to prepare cultures in the usual manner with the secondary stimulus being effected on day 0 in vitro. On day 15 of the cultures' life, when antibody synthesis was greatest, replacement medium was added which contained either C^{14}-algal protein hydrolysate or C^{14}-tyrosine. The former radioactive label was used in the experiment shown in Fig. 3. In the culture illustrated here, the radioactive medium was replaced every 2 hr over the next 24 hr, and the fluids removed were saved. Antibody in these fluids was coprecipitated to relatively constant specific radioactivity by a method described elsewhere (12*a*). Isotopically labeled antiribonuclease was detected in the fluid removed from this culture after the first 2 hr of incubation, thus indicating a transit time for antibody in this system of less than 2 hr. Furthermore, incorporation of the labeled amino acids into antibody reached a maximal level within 8 hr after the first addition of the C^{14}-hydrolysate.

The experiment with puromycin (Fig. 2) also indicated that this culture system, unlike bacteria, is almost irreparably damaged by this agent. As mentioned above, the addition of 10 μM and 3 μM puromycin promptly terminated antibody synthesis, while 1 μM had negligible or no immediate effect. Thus, 3 μM is an adequate but not excessively high inhibitory concentration. When 3 μM puromycin was included in the medium only from day 9 to day 12, and the cultures were then rinsed three times before continuing their incubation, they did not resume antibody production. When cultures were exposed to the same concentration for only 1 day and then rinsed three times, there was a slight return of antibody production. The failure of these cultures to recover significantly from a pulse of puromycin indicates how sensitive this system is to a metabolic inhibitor which acts at a terminal step in protein production. This apparent lability has prompted us in studies with other inhibitors to investigate generally the effects of minimally suppressive concentrations. That is, the concentration studied for most inhibitors is the lowest one which depresses the antibody response to less than 10% of the control value when the agent is present in the medium from day 0 through the end of the experiment.

INHIBITION BY CHLORAMPHENICOL AND ACTINOMYCIN D

The first compound we examined several years ago was chloramphenicol (1). When this work was started in 1960, it was commonly thought

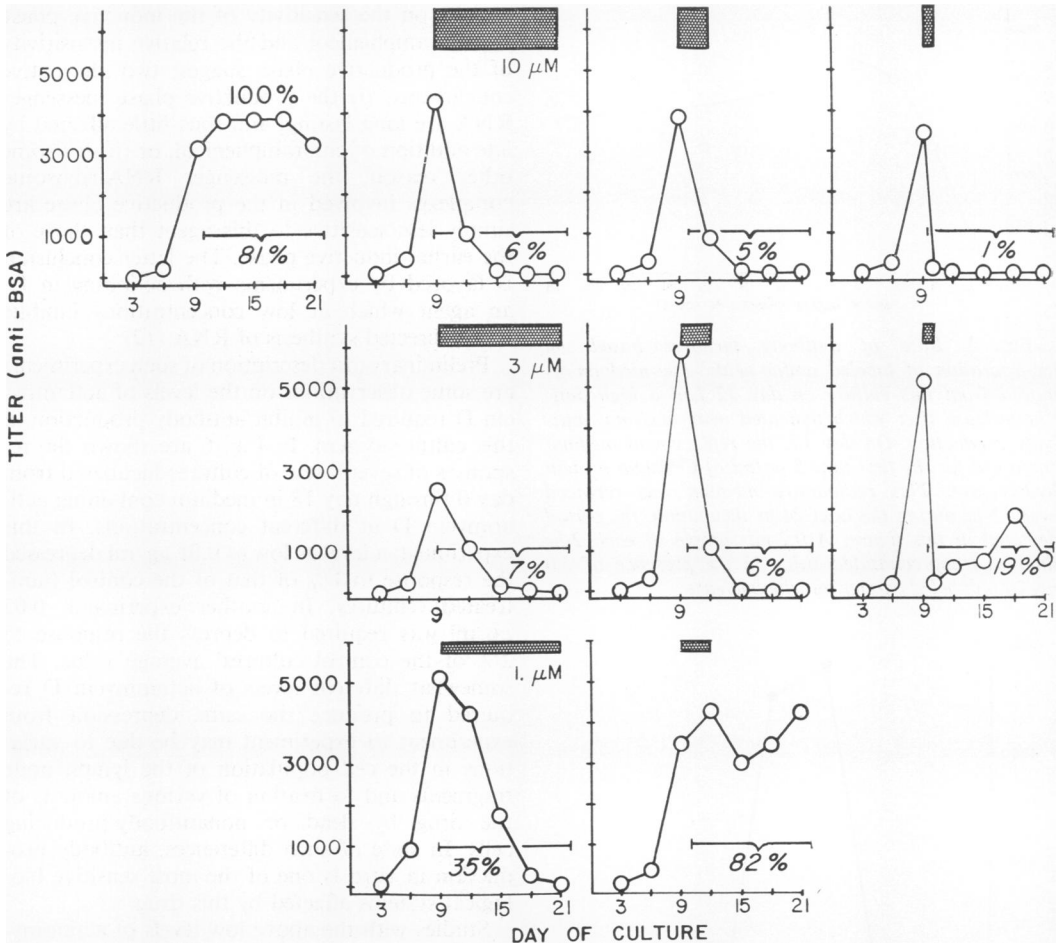


FIG. 2. *Inhibition of the secondary antibody response by puromycin. The stippled bar above each graph denotes the interval of treatment with this drug; concentrations of 1, 3, and 10 μM were used, as indicated under the first bar in each row of graphs. When puromycin treatment was discontinued on day 12 or on day 10, the affected cultures were then rinsed three times in regular medium before their incubation was continued. The slanted percentage figures represent the amount of antibody produced after day 9 relative to the total 21-day production in the control set of cultures. There were four cultures in the control group and three in each of the other groups.*

that bacteriostatic levels of chloramphenicol did not inhibit protein synthesis in mammalian cells (21). However, we found that as little as 5 μg/ml (Fig. 4) significantly depressed the secondary response in vitro, and that 50 μg/ml often abolished it completely. The interesting feature of this inhibition is illustrated in Fig. 5 and involves two observations. First, treatment of cultures with an inhibitory level of the drug (50 μg/ml) for the first 6 days depressed the response as completely as did treatment throughout the duration of the experiment. Second, the addition of this inhibitory concentration to cultures starting on day 6 caused inhibition which was disproportionately small, in view of the fact that most of the antibody was elaborated after day 6 in this experiment. At the time, these two observations led us to regard the inductive phase as the major focus of chloramphenicol's action. Other considerations prompted us to speculate that the drug interferes with messenger ribonucleic acid (RNA) function. Weisberger and co-workers have since presented evidence in a cell-free system that chloramphenicol blocks the attachment of messenger RNA to rabbit reticulocyte ribosomes (22).

In our culture system, it is difficult to establish that an agent affects a particular messenger RNA, since many different messenger RNA are involved, not only the one (or several) responsible for final antibody synthesis, but also others which direct the synthesis of cellular proteins necessary

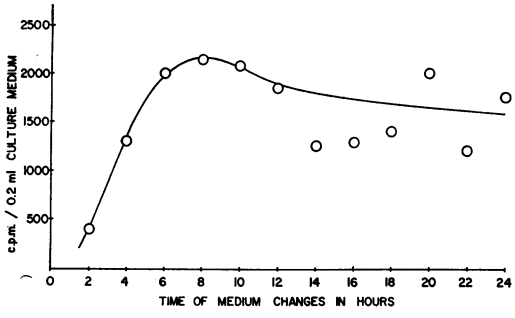


FIG. 3. *Rate of antibody turnover based on incorporation of labeled amino acids. The medium removed from this culture on day 12 had a high antiribonuclease titer which indicated near maximal antibody production. On day 15, the replacement medium contained for the first time 5 μc/ml of C^{14}-algal protein hydrolysate. This radioactive medium was replaced every 2 hr during the next 24-hr incubation, the period depicted in this figure. A 0.2-ml sample of each 2-hr fluid was coprecipitated twice in the presence of 2.0 mg of unlabeled carrier antiribonuclease.*

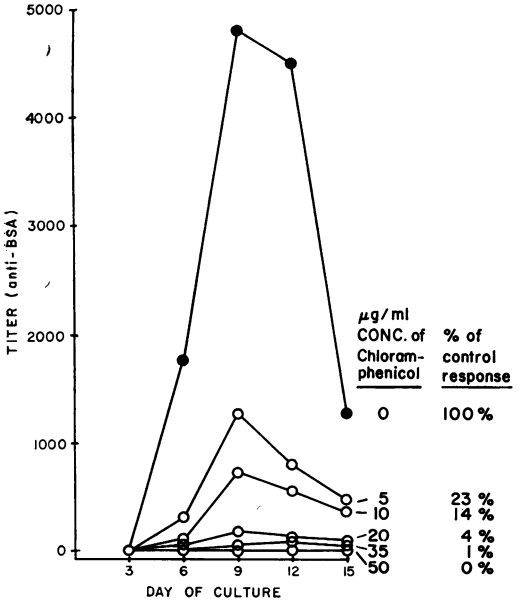


FIG. 4. *Inhibition of the secondary antibody response by chloramphenicol. Cultures were treated for 15 days with chloramphenicol at concentrations between 5 and 50 μg/ml. (The medium consisted of Eagle's MEM supplemented with 25% normal rabbit serum.) There were four cultures for each variable.*

at different stages for multiplication, differentiation, and simple maintenance. If the precise mechanism proposed by Weisberger's group also applies to intact lymphoid cells, then our observations on the sensitivity of the inductive phase to chloramphenicol and the relative insensitivity of the productive phase suggest two alternative conclusions: (i) the productive phase messenger RNA are long lasting, and thus little affected by late addition of chloramphenicol, or (ii) for some other reason, the messenger RNA-ribosome complexes involved in the productive phase are simply less sensitive to this agent than those of the earlier inductive phase. The latter conclusion is favored by experiments with actinomycin D, an agent which at low concentrations inhibits DNA-directed synthesis of RNA (12).

Preliminary to a description of such experiments are some observations on the levels of actinomycin D required to inhibit antibody production in this culture system. In Fig. 6 are shown the responses of several sets of cultures incubated from day 0 through day 18 in medium containing actinomycin D at different concentrations. In this experiment, a level as low as 0.01 μg/ml depressed the response to 1% of that of the control (nontreated) cultures. In another experiment, 0.02 μg/ml was required to depress the response to 2% of the control cultures' average value. The somewhat different levels of actinomycin D required to produce the same depression from experiment to experiment may be due to variations in the cell population of the lymph node fragments and to fixation of various amounts of the drug by dead or nonantibody-producing cells. In spite of such differences, antibody production in vitro is one of the most sensitive biological systems affected by this drug.

Studies with the above low levels of actinomycin D suggest that messenger RNA formed during the productive phase of antibody synthesis is not long lasting, but functions for only several days. This observation is illustrated in Fig. 7, which contrasts the effect on antibody production of treatment with puromycin, actinomycin D, or chloramphenicol starting on day 9. Media were replaced daily in these cultures, and the cumulative average titer for each group was plotted. The control (untreated) group continued producing antibody at a steady rate through day 21. However, in another group, this production was stopped within 1 day of the start of puromycin treatment on day 9. (This response to puromycin eliminates, in this experiment, any concern for secretion of "preformed" antibody into later medium changes.) A somewhat slower termination of synthesis was produced by the start of actinomycin D treatment also on day 9; in this group of cultures, antibody synthesis continued at the control rate for 3 to 4 days and then abruptly stopped around day 14. But in the chloramphenicol-treated group, antibody pro-

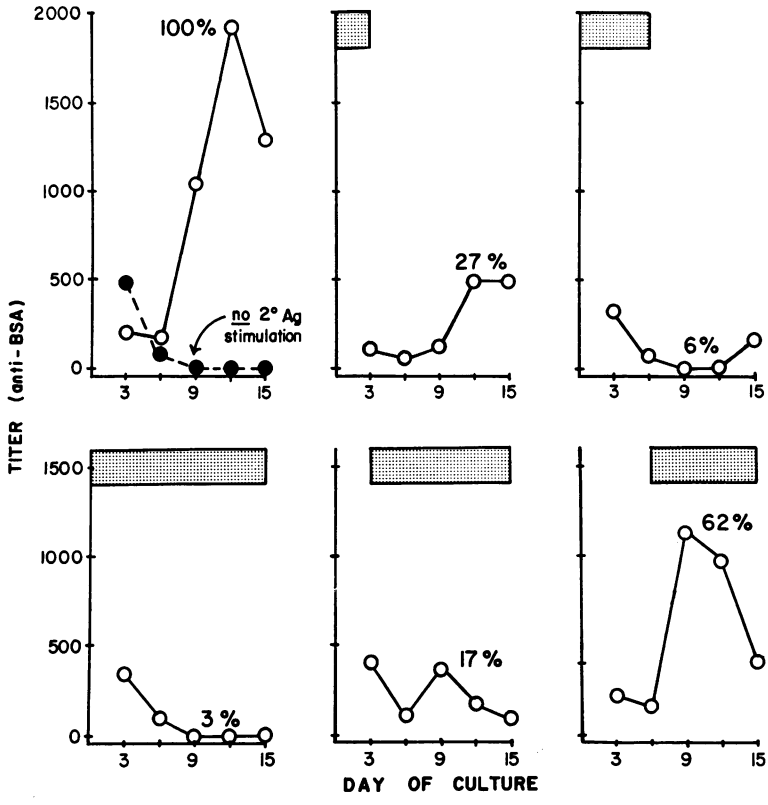


FIG. 5. *Temporal effect of chloramphenicol on the secondary antibody response. The stippled bar above each graph denotes the interval of treatment with 50 μg/ml of chloramphenicol. (The medium consisted of Eagle's MEM supplemented with 25% normal rabbit serum.) There were three cultures in each group.*

duction continued through day 21 at the same rate as in the control group, indicating the ineffectiveness of this drug when added to cultures starting on day 9.

We assume the inhibition produced by actinomycin D in this experiment correctly reflects the functional longevity of messenger RNA coded for antibody synthesis. An agent, such as chloramphenicol, which presumably interferes with the attachment of new messenger RNA to ribosomes, might be expected to have the same effect on the rate of antibody synthesis as does actinomycin D, which stops production of new messenger RNA. But chloramphenicol treatment throughout day 9 to 21 did not cause inhibition of antibody synthesis paralleling that caused by an identical period of actinomycin D treatment. Several hypotheses can be advanced to explain this puzzling difference; the most general one is that messenger RNA complexes involved in the productive phase are simply less vulnerable to chloramphenicol's inhibition than those responsible for the earlier inductive phase. Possibly the families of ribosomes functioning during these two phases of the immune response differ in their sensitivity to chloramphenicol.

INHIBITION BY SALICYLATE

Another compound which exerts an inhibitory effect on the immune response primarily during its inductive phase is salicylate (4). Interest in salicylate and aspirin as possible immunosuppressant drugs flourished a few decades ago, but lapsed after several equivocal clinical and experimental reports [reviewed in (5, 14)]. However, Fig. 8 shows that sodium salicylate clearly inhibits the secondary antibody response in vitro at concentrations between 0.5 and 1.5 mM. The translation of this range effective in vitro to comparable blood levels in man and experimental animals must take into consideration two facts: (i) salicylate binds to plasma proteins [about 75% is bound at low therapeutic blood levels, and 50% at high levels (15)] and (ii) the therapeutic activity is determined by the unbound form of the drug and not its total concentration (16). The range of 0.5 to 1.5 mM is equivalent to 8 to 24 mg/100 ml of nonprotein-bound (free)

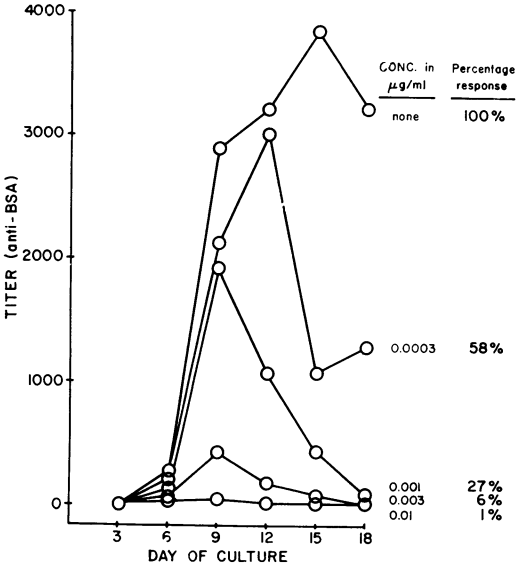


FIG. 6. *Inhibition of the secondary antibody response by actinomycin D. Cultures were treated for 18 days with actinomycin D at concentrations between 0.0003 and 0.01 μg/ml. There were four cultures in the control group and three in each of the other groups.*

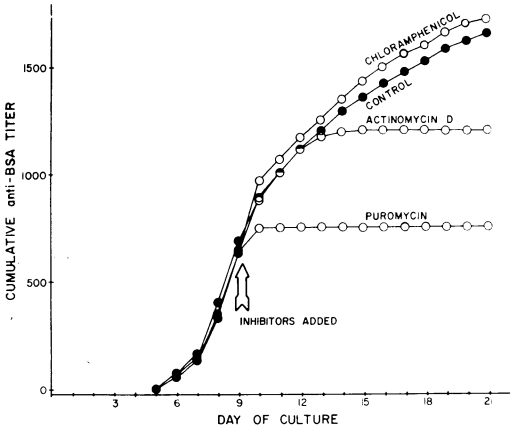


FIG. 7. *Comparative inhibition of the productive phase of the secondary antibody response by puromycin, actinomycin D, and chloramphenicol. The media of these cultures were replaced daily, as indicated along the abscissa. The ordinate shows the cumulative average antibody titers for each of the four culture groups: each contained four culture tubes. The closed circles (—●—) represent the average titers of the control (untreated) group and of the other three groups prior to the addition of the various metabolic inhibitors on day 9. The open circles (—○—) represent the average titers of the treated groups whose media contained after day 9 either 2.7 μg/ml (5 μM) of puromycin, 0.02 μg/ml of actinomycin D, or 40 μg/ml (125 μM) of chloramphenicol.*

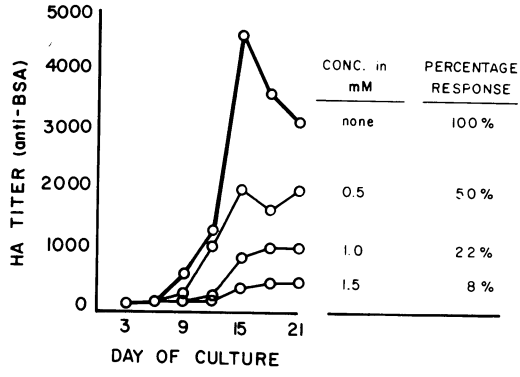


FIG. 8. *Inhibition of the secondary antibody response by salicylate. Cultures were treated for 21 days with sodium salicylate at concentrations between 0.5 mM and 1.5 mM. There were five cultures in the control group and four in each of the other groups.*

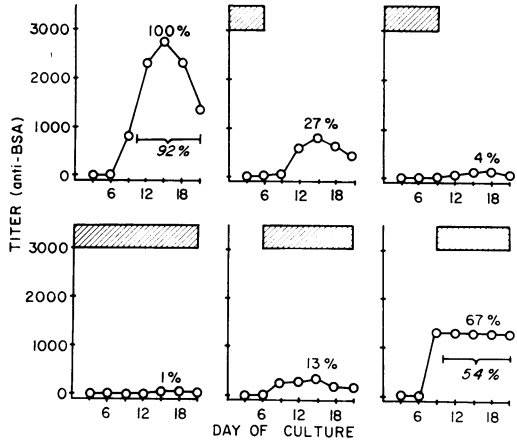


FIG. 9. *Temporal effect of salicylate on the secondary antibody response. The vertically hatched bar above each graph indicates the interval of treatment with 1.5 mM sodium salicylate. The percentage figure above each response curve denotes the total average antibody production in that group; the slanted percentage figure beneath several curves denotes the average antibody production for that group after day 9.*

salicylate in the blood and is approximately represented by 30 to 50 mg/100 ml of total salicylate. In patients intensively treated with aspirin, total blood levels above 30 mg/100 ml can readily be sustained, and peaks up to 50 mg/100 ml and higher are often reached before toxic manifestations develop (15).

An experiment demonstrating the temporal effect of salicylate suppression in vitro is shown in Fig. 9. Medium containing 1.5 mM sodium salicylate was added to groups of cultures for different time intervals, indicated by the cross-hatched bar above each graph. In the control

group maintained for 21 days, 92% of the total antibody was produced after day 9. In other cultures treated with salicylate for the entire 21 days, only a 1% response occurred. Treatment for only the first 9 days depressed the response to 4% and was thus almost as effective as was treatment for 21 days. On the other hand, cultures treated with salicylate from day 9 through day 21 produced an average response of 67%, which is quite significant, considering that 92% of the total antibody in the control group was produced after day 9. These data indicate that the inductive phase is more vulnerable than the productive phase to salicylate inhibition and suggest that this drug should be carefully re-examined in vivo as a potentially useful immunosuppressive agent.

REQUIREMENT FOR CORTICOSTEROIDS

This discussion so far has concerned two agents which inhibit preferentially metabolic steps in the inductive phase. The opposite effect, namely, the promotion or support of the inductive phase, is mediated in vitro by certain adrenocortical hormones. Several years ago we found that cortisol (hydrocortisone) can replace serum in the medium of this system (2). Figure 10 demonstrates that the secondary response is supported by 0.01 to 10 μM sodium cortisol hemisuccinate ("Solu-Cortef," The Upjohn Co., Kalamazoo, Mich.) in a serum-free medium. Medium with a lower concentration or none at all failed to support antibody production, and concentrations above 10 μM became inhibitory. A similar range of effective concentrations also pertains for other forms of cortisol, for cortisone (which is converted to cortisol), for corticosterone (the major adrenal steroid secreted by rabbits), and several other naturally occurring corticosteroids (3). Rabbit plasma normally contains around 0.1 μM cortisol and 0.2 to 0.3 μM corticosterone (20). Since these physiological levels are highly effective in supporting antibody production in vitro, we suspect that corticosteroids serve a similar function in vivo.

The particular relevance of cortisol to this discussion is the observation that it is necessary for some process which occurs during the inductive phase (Fig. 11). In the experiment depicted here, cortisol was included in the serum-free culture medium for only certain periods, as indicated by the stippled bar across the top of each graph. A minimally effective level of cortisol (0.01 μM) was used so that, when the steroid was discontinued in the medium on day 3, 6, or 9, several rinses of a culture would reduce at least the extracellular cortisol to an ineffective level. The 100% reference response was produced by cultures incubated for 21 days in cortisol-containing medium. Cultures never exposed to cortisol produced essentially no response. The presence of cortisol for the first 3 days supported only a 30% response, for the first 6 days, a 55% response, and, for the first 9 days, a response as good as that of the control group. On the other hand, when cortisol was included in the medium starting on day 6, only a 13% response was found, and, on day 9, only a 3% response. Thus, the inductive phase appears to be the focus of cortisol's action.

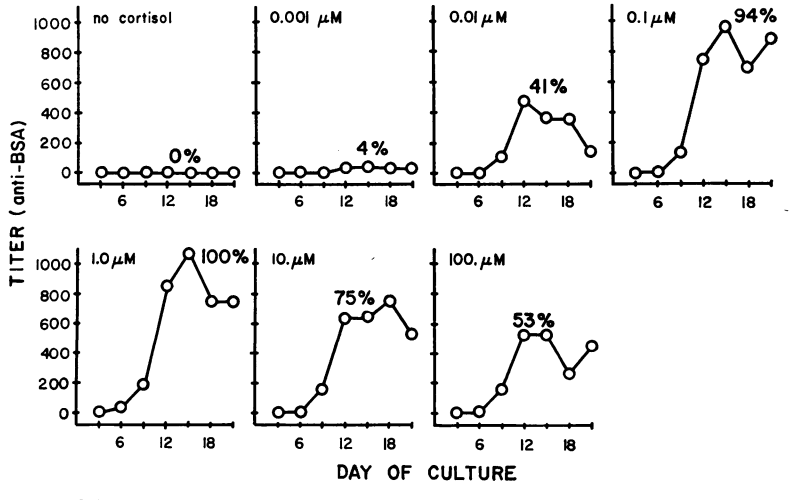


FIG. 10. *Support of the secondary response by different concentrations of cortisol. Groups each consisting of four cultures were incubated in serum-free medium containing between 0.001 and 100 μM sodium cortisol hemisuccinate ("Solu-Cortef," The Upjohn Co., Kalamazoo, Mich.). The response in medium containing 1.0 μM cortisol was taken as the 100% reference.*

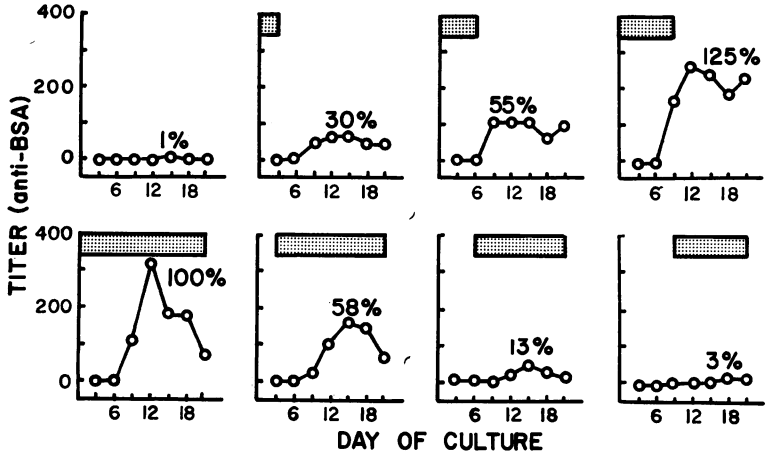


FIG. 11. *Temporal effect of cortisol on the secondary antibody response. The stippled bar above each graph indicates the interval of treatment with serum-free medium containing 0.01 μM sodium cortisol hemisuccinate. When the cortisol was discontinued in the medium on day 3, 6, or 9, the affected cultures were rinsed three times in cortisol-free, serum-free medium before their incubation was continued in this rinse medium. The other cultures were also subjected to this rinsing procedure as a control measure. There were four cultures in each group.*

INHIBITION BY OTHER STEROID HORMONES

While surveying other steroids for their ability to duplicate cortisol's supportive effect, we observed that 1.0 to 10 μM testosterone, estradiol (both 17α- and 17β-), progesterone, or 17α-hydroxyprogesterone inhibited the secondary response. The temporal pattern of this inhibition is shown in Fig. 12. The top two graphs demonstrate the effect previously described, namely, that the presence of cortisol in the serum-free medium for the first 9 days supports a response equal to that when cortisol is present for the entire 21 days. The bottom two graphs show that 10 μM testosterone added on day 0 almost completely suppressed this response, but that, when added on day 9, this concentration had no inhibitory effect. Again, these data implicate the inductive phase as the general point of inhibition by testosterone. In other experiments, progesterone exhibited a similar temporal pattern of suppression. We have also noted that the inhibition produced by these two hormones can partly be overcome by raising the concentration of cortisol in the medium. This fact, plus the temporal similarity in their opposing effects, raises the possibility that the inhibitory effect of testosterone and progesterone is merely due to competition with cortisol for a common reactive site within the antigen-stimulated cells.

Most of the compounds discussed here (chloramphenicol, salicylate, cortisol, progesterone, and testosterone) are new to the in vitro examination of the immune response. Further study of these agents in this system may reveal more precisely the metabolic steps they each affect.

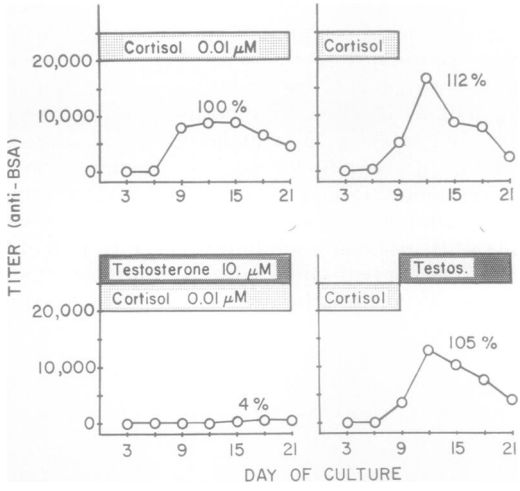


FIG. 12. *Temporal effect of testosterone on the secondary antibody response. The bars above each graph indicate the interval of treatment with 0.01 μM cortisol and 10 μM testosterone. There were four cultures in each group.*

ACKNOWLEDGMENTS

I thank Albert H. Coons for his constant encouragement and unstinting support throughout the 4 years of this work. I gratefully acknowledge also the assistance during this time of a succession of exceptionally competent and devoted technicians, including Linda Santoro, Peter M. Fitz, Shirley Stein, Elsa Meekins, and Angela Lamb.

This investigation was supported by Public Health Service grants H-2255 and AI-05691 from the National Institute of Allergy and Infectious Diseases.

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